Helicobacter pylori infection can cause duodenal ulcers and may also induce gastric adenocarcinoma. The bacteria colonize the gastric mucosa and areas of gastric metaplasia in the duodenum for decades, resulting in active chronic inflammation in the infected areas. A characteristic feature of the infection is the ongoing recruitment of neutrophils to the infected sites. To evaluate the role of H. pylori lipopolysaccharides (LPS) in the recruitment of leukocytes to the gastric mucosa, we have examined the cytokine and chemokine production from human monocytes stimulated with LPS isolated from different H. pylori strains, as well as from several other gram-negative bacteria. Our results show that H. pylori LPS induce a large production of neutrophil-recruiting CXC chemokines (interleukin-8 and growth-related oncogene alpha) from purified human monocytes, to almost the same extent as Escherichia coli LPS. However, and in agreement with previous studies, H. pylori LPS was much less potent in inducing production of proinflammatory cytokines by purified human monocytes and was also a weak inducer of the CC chemokine RANTES. There was no difference between LPS preparations from different H. pylori strains in their ability to induce cytokines and chemokines. The preferential production of CXC chemokines after stimulation with H. pylori LPS indicates an important contribution of this molecule in maintaining neutrophil recruitment during the infection, irrespective of the infecting strain.

Infection with the gram-negative bacterium Helicobacter pylori is associated with development of gastric and duodenal ulcers and in some instances also with gastric carcinoma (7). H. pylori infection is characterized by an active chronic gastric inflammation with invasion of polymorphonuclear as well as mononuclear cells. A local increase in the expression of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α), as well as high levels of the neutrophil-recruiting chemokine IL-8, in the H. pylori-infected stomach has also been documented (15, 30).

Infection with H. pylori does not always give rise to gastric symptoms. Approximately 10 to 15% of infected individuals develop dyspepsia or peptic ulcers, whereas the rest remain more or less asymptomatic (39). The factors that determine the outcome of an H. pylori infection are still relatively unknown (43). There are indications that H. pylori strains differ in their ability to induce cytokine production; e.g., an important feature of the bacteria for the clinical outcome of the infection seems to be the presence of the cytotoxin-associated gene A (cagA) and the cagA-associated ice gene, which are associated with ulcer disease (18, 32, 41, 46). Furthermore, studies from our laboratory and others have shown that other putative virulence factors, e.g., expression of the adhesin BabA on certain Lewis antigens, are associated with development of a symptomatic H. pylori infection (41, 46). However, host factors also seem to be important for the clinical outcome, since studies with mice and humans have shown different outcomes of the infection depending on the genetic background of the host (13, 17, 34).

It is well known that bacterial lipopolysaccharides (LPS) may induce both strong local and systemic inflammation in animals as well as humans, and therefore, H. pylori LPS is one of the factors that could potentially influence local gastric inflammation and the clinical outcome during an H. pylori infection. In general, H. pylori LPS is much less potent in activation of inflammatory cells than LPS from members of the family Enterobacteriaceae, e.g., Escherichia coli and Salmonella spp. (5, 24, 26). This may be explained by the structural differences between the lipid A molecules of LPS from H. pylori and the Enterobacteriaceae. Another unusual feature of H. pylori LPS is the presence of Lewis blood group antigens on the carbohydrate side chains (2, 3, 10, 25, 26). In spite of its relatively low toxic activity, H. pylori LPS has been shown to activate inflammatory cells to produce different cytokines and chemokines, such as TNF-α, IL-8, IL-1, and monocyte chemotactic protein-1 (5). Nevertheless, the potential capacity of purified H. pylori LPS to induce chemokine production has not been extensively studied. Furthermore, it is not known whether LPS from different H. pylori strains may differ in their capacities to induce cytokine and chemokine production.

Therefore, we have examined the cytokine and chemokine responses induced by H. pylori LPS purified from bacteria isolated from asymptomatic carriers and duodenal ulcer patients, as well as LPS preparations expressing different Lewis blood group antigens. These responses were compared to those induced by LPS from several other gram-negative species.

**MATERIALS AND METHODS**

LPS preparations. H. pylori bacteria from strains Hel 73, Hel 230, Hel 234, and Hel 255 and Sydney strain 1 (SS1) were cultured on horse blood agar plates. The different strains were selected based on whether they were isolated from patients with duodenal ulcers or from asymptomatic carriers as well as on the type of Lewis antigens expressed on their LPS (Table 1). SS1 was originally provided by Adrian Lee, Sydney, Australia (23) and had been adapted to growth in mice by several passages. All the other strains had been isolated in our laboratory from

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Swedish volunteers undergoing gastroscopy for diagnostic purpose. After 3 days of culture on horse blood agar plates in a microaerophilic milieu (i.e., 10% CO₂, 5% O₂, and 85% N₂) at 37°C, the bacterial cells were harvested and LPS was prepared by the hot-phenol-water method described by Westphal and Jann (44), dialyzed, and freeze-dried. The final product was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining and was shown to give a typical ladder formation (42). The protein content was spectrophotometrically measured and shown to be less than 5% (wt/wt). In preliminary experiments LPS preparations that had been further purified by treatment with proteases, RNase, and DNase (42) gave rise to cytokine responses identical to those of the crude LPS preparations (data not shown). Therefore, the crude LPS preparations were used in the rest of the experiments. LPS isolated from the bacterium *Salmonella enterica* serovar Newport, *Vibrio cholerae* strain 569B, *E. coli* strain H10407, and *Haemophilus ducreyi* strain 4747 (kindly provided by T. Lagergård) and prepared according to the same procedure were also included in the study.

**Demonstration of Lewis blood group antigens on *H. pylori*.** The *H. pylori* bacteria were characterized for Lewis blood group antigen (Le⁰, Le⁺, Le², and Le³) expression in the LPS by use of a slightly modified whole-cell enzyme-linked immunosorbent assay (37, 41). Briefly, 100-μl portions of previously frozen bacteria diluted to an optical density (OD) of 0.2 at 600 nm, corresponding to 10⁷ bacteria/ml, were incubated in 96-well Maxisorb (Nunc, Roskilde, Denmark) plates at 4°C overnight. After being blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), monoclonal antibodies (Signet Laboratory, Dedham, Mass.) against the different Lewis antigens diluted 1/1,500 in BSA-PBS-Tween were added to each well. After incubation for 90 min at 37°C, plates were developed with a horseradish peroxidase-conjugated goat antibody to mouse immunoglobulins (Jackson Immuno Research Laboratories Inc., West Grove, Pa.) followed by addition of H₂O₂ and o-phenylenediaminedichromide (OPD) as a chromogen. The plates were read at 450 nm in a spectrophotometer (Multiscan MS; Labsystems) at 492 nm. The same procedure was used when analyzing all cytokines and chemokines. R&D Systems reagents were obtained from Pharmingen. Standard curves were constructed in triplicate and were incubated for 48 h. In some experiments, the produced TNF-α was neutralized by adding a mouse monoclonal antibody to human TNF-α (Pharmingen, Becton Dickinson, Stockholm, Sweden) at an antibody concentration of 2 μg/ml, a concentration that has previously been shown to inhibit the effect of 100 ng of TNF-α per ml on endothelial cell production of IL-8. Nonstimulated PBMC, monocytes, or macrophages were used as controls. The supernatants were aliquoted and stored at −80°C until cytokine analysis. All buffer and media used in the experiment were tested and found to contain <0.03 endotoxin unit of endotoxin per ml using the Limulus test.

**Detection of cytokines and chemokines.** The levels of IL-8, TNF-α, IL-10, growth-related oncogene alpha (GRO-α), RANTES, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the cell supernatants were determined using the enzyme-linked immunosorbent assay technique. For the detection of IL-8, 96-well Maxisorb (Nunc) plates were coated with mouse anti-human IL-8 (R&D Systems, Oxon, United Kingdom) and incubated overnight at 4°C. The plates were blocked with PBS containing 1% BSA (Sigma-Aldrich), and samples were incubated at 4°C overnight. Biotinylated rabbit anti-human IL-8 antibodies (R&D Systems), followed by extravidin-horseradish peroxidase (Sigma-Aldrich), were used for detection, employing hydrogen peroxide and OPD as substrates. After addition of 25 μl of 1 M H₂SO₄, the final reaction was measured by a spectrophotometer (Multispec MS; Labsystems) at 492 nm. The same procedure was used when analyzing all cytokines and chemokines. R&D Systems produced the GM-CSF, GRO-α, and RANTES reagents. IL-10 and TNF-α reagents were obtained from Pharmingen. Standard curves were constructed using recombinant cytokines, and the detection limits were 15.6 pg/ml for IL-8; 31.2 pg/ml for GM-CSF, RANTES, and IL-10; 78.1 pg/ml for TNF-α; and 312 pg/ml for GRO-α.

**Statistical analysis.** Differences were evaluated by use of one-way analysis of variance (ANOVA) (Kruskall-Wallis) followed by Dunn’s multiple-comparison test.

**RESULTS**

**Cytokine production by *H. pylori* after LPS stimulation.** Stimulation of PBMC from four individuals with LPS (10 μg/ml) from *H. pylori* Hel 73, *E. coli* H10407, *S. enterica* serovar Newport, *V. cholerae* 569B, and *H. ducreyi* resulted in production of IL-8. Stimulation with *H. pylori* LPS induced PBMC to produce IL-8 to the same extent as the other LPS preparations used (Fig. 1). In contrast, *E. coli*, and *S. enterica* serovar Newport LPS induced 3.5 to 6 times more IL-10 than *H. pylori* or *V. cholerae* LPS (Fig. 1). Likewise, a relatively low production of TNF-α was seen when PBMC were stimulated with *H. pylori* LPS (Fig. 1) compared to the other LPS preparations. CD14⁺ cells are the main source of cytokines after stimulation with *H. pylori* LPS. In order to determine which cell types in the PBMC suspension responded to *H. pylori* LPS, selected cell subsets were depleted from the PBMC suspension containing 5% human AB+ serum, 1% l-glutamine, and 1% gentamicine (Isco’s complete medium) at a final concentration of 5 × 10⁵ monocytes/ml. One hundred microliters of the suspension was incubated in 96-well plates for 2 h at 37°C in 5% CO₂. After incubation, the wells were repeatedly washed with PBS with 1% human AB+ serum. The supernatants were collected again and analyzed by flow cytometry to confirm the adherence of the monocytes to the cell culture plate. To the remaining adherent monocytes 100 μl of Isco’s complete medium was added, and the plates were kept at 37°C in 5% CO₂. The monocytes were either stimulated immediately or allowed to differentiate to macrophages by further incubation at 37°C in 5% CO₂ for 1 week (20). Differentiation to macrophages was confirmed by analysis of myeloperoxidase content, which is lost during maturation from monocytes to macrophages (20). Macrophages and monocytes were lysed by use of 0.02% Triton X-100 in PBS, and myeloperoxidase was determined by addition of OPD (Sigma-Aldrich, Stockholm, Sweden) (20). Following 1 week of differentiation, the macrophages were stimulated with LPS, and at the same time the old medium was replaced with 100 μl of fresh Isco’s complete medium.

**Stimulation of PBMC subsets.** In initial experiments unfraccionated PBMC and cell suspensions depleted of different lymphocyte subsets (2 × 10⁶ cells) were stimulated with LPS (10 μg/ml) from *H. pylori* Hel 73, *E. coli*, *S. enterica* serovar Newport, *V. cholerae*, and *H. ducreyi* for 24 h. Purified monocytes and macrophages were stimulated with LPS (2.5 or 25 μg/ml) from *H. pylori* SS1, Hel 73, Hel 230, Hel 234, and Hel 255, E. coli, *S. enterica* serovar Newport, *V. cholerae*, and *H. ducreyi* in triplicate and were incubated for 48 h. In some experiments, the produced TNF-α was neutralized by adding a mouse monoclonal antibody to human TNF-α (Pharmingen, Becton Dickinson, Stockholm, Sweden) at an antibody concentration of 2 μg/ml, a concentration that has previously been shown to inhibit the effect of 100 ng of TNF-α per ml on endothelial cell production of IL-8. Nonstimulated PBMC, monocytes, or macrophages were used as controls. The supernatants were pooled, aliquoted, and stored at −80°C until cytokine analysis. All buffer and media used in the experiment were tested and found to contain <0.03 endotoxin unit of endotoxin per ml using the Limulus test.

**Detection of cytokines and chemokines.** The levels of IL-8, TNF-α, IL-10, growth-related oncogene alpha (GRO-α), RANTES, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the cell supernatants were determined using the enzyme-linked immunosorbent assay technique. For the detection of IL-8, 96-well Maxisorb (Nunc) plates were coated with mouse anti-human IL-8 (R&D Systems, Oxon, United Kingdom) and incubated overnight at 4°C. The plates were blocked with PBS containing 1% BSA (Sigma-Aldrich), and samples were incubated at 4°C overnight. Biotinylated rabbit anti-human IL-8 antibodies (R&D Systems), followed by extravidin-horseradish peroxidase (Sigma-Aldrich), were used for detection, employing hydrogen peroxide and OPD as substrates. After addition of 25 μl of 1 M H₂SO₄, the final reaction was measured in a spectrophotometer (Multispec MS; Labsystems) at 492 nm. The same procedure was used when analyzing all cytokines and chemokines. R&D Systems produced the GM-CSF, GRO-α, and RANTES reagents. IL-10 and TNF-α reagents were obtained from Pharmingen. Standard curves were constructed using recombinant cytokines, and the detection limits were 15.6 pg/ml for IL-8; 31.2 pg/ml for GM-CSF, RANTES, and IL-10; 78.1 pg/ml for TNF-α; and 312 pg/ml for GRO-α.

**Statistical analysis.** Differences were evaluated by use of one-way analysis of variance (ANOVA) (Kruskall-Wallis) followed by Dunn’s multiple-comparison test.
with Dynabeads coated with different anti-CD antibodies and the remaining cells were stimulated with LPS (10 μg/ml) from *H. pylori* Hel 73, *E. coli* H10407, *S. enterica* serovar Newport, *V. cholerae*, and *H. ducreyi* for 48 h is shown. Bars indicate geometric means + standard errors of the means. Statistical evaluations of differences between control and stimulated samples were performed by one-way ANOVA (Kruskall-Wallis) followed by Dunn's multiple-comparison test.

FIG. 1. Cytokine production by PBMC after LPS stimulation. Production of IL-8, IL-10, and TNF-α from PBMC stimulated with LPS (10 μg/ml) from *H. pylori* Hel 73, *E. coli* H10407, *S. enterica* serovar Newport, *V. cholerae*, and *H. ducreyi* for 48 h is shown. Bars indicate geometric means + standard errors of the means. Statistical evaluations of differences between control and stimulated samples were performed by one-way ANOVA (Kruskall-Wallis) followed by Dunn's multiple-comparison test.

on a per-cell basis, which could depend on some regulatory effect exerted by these cells. In this particular set of volunteers *H. pylori* LPS induced even higher levels of IL-8 production than *E. coli* LPS, while *E. coli* LPS was a more potent inducer of IL-10 and TNF-α production.

**LPS from different *H. pylori* strains induce similar responses.** Based on the depletion experiments, we used purified
monocytes when comparing the effects of LPS isolated from different *H. pylori* strains. These were initiated with titration experiments to determine the optimal conditions for the LPS stimulation in our systems. We found that 25 μg of *H. pylori* LPS per ml yielded much higher cytokine responses than 2.5 μg of *H. pylori* LPS per ml. Cytokine levels were maximal after 48 h of LPS stimulation. Unstimulated monocytes were used as controls in all of the following experiments.

To determine if there were any differences between LPS preparations from different *H. pylori* strains, we compared cy-

FIG. 2. Depletion of leukocyte subsets from PBMC before LPS stimulation. Production of IL-8, IL-10, and TNF-α from PBMC with selected cell types depleted after stimulation with 10 μg of *H. pylori* Hel 73 LPS (closed bars) or *E. coli* H10407 LPS (open bars) per ml is shown. Bars indicate geometric means ± standard errors of the means.
In this study we show that *H. pylori* LPS can induce high levels of CXC chemokines in spite of its comparatively low capacity to induce production of proinflammatory cytokines. In several previous studies *H. pylori* LPS has been shown to be less potent in activating the immune system than LPS from other gram-negative intestinal bacteria (i.e., *Enterobacteriaceae*) (5, 16, 21, 24, 27, 33). These findings are confirmed by the present study, which shows considerably lower levels of TNF-α after stimulation with *H. pylori* LPS than after stimulation with *E. coli* LPS. Furthermore, we show that the inflammation-downregulatory cytokine IL-10, as well as GM-CSF, is produced to a much smaller extent after stimulation with *H. pylori* LPS than after stimulation with LPS isolated from other bacterial species. Blocking experiments demonstrate that the IL-10 and GM-CSF production resulting from stimulation with *E. coli* LPS was only partly caused by autocrine actions of TNF-α. Therefore, the low ability of *H. pylori* LPS to induce these cytokines reflects actual differences between the LPS preparations examined and stands in contrast to the effects of LPS from all other species used in this study. The low production of downregulatory cytokines may perhaps also contribute to the continuous inflammation seen during *H. pylori* infection.

*H. pylori* LPS binds to CD14 (21), like LPS from other species, but many of the intracellular responses seen after *H. pylori* LPS stimulation, such as NF-κB and protein kinase C activation, are mediated by LPS-independent pathways (29, 40). The low level of inflammation induced by *H. pylori* LPS has even been speculated to be beneficial for bacterial persistence (6, 8), since *H. pylori* infection might not induce strong enough responses for the host to eradicate the bacteria.
The relatively low stimulatory activity of *H. pylori* LPS could be due to structural differences, such as different phosphorylation patterns and an unusual fatty acid composition of the lipid A part compared to those in LPS from other bacteria (16, 27, 28, 31, 38). Another potentially important feature is that different *H. pylori* strains modify their LPS by variable expression of the human Lewis blood group antigens on the oligosaccharide structure (1, 10, 25, 26). Indeed, certain Lewis antigens have been associated with duodenal ulcer disease (41, 46). There was, however, no difference in levels of cytokine or chemokine production after stimulation with LPS isolated from strains with different Lewis blood group antigens or from strains from patients with different symptoms, suggesting that it is the conserved lipid A portion or other regions of the LPS that are responsible for the activity. Studies using LPS from different strains of *Salmonella* spp. have suggested a possibility
that rough strains have a greater capacity to induce inflammation than smoother strains. However, in preliminary studies we did not find any differences in the stimulatory activities of LPS prepared from the rough strain CCUG 17874 (42) and the other \textit{H. pylori} LPS used (M. Innocenti, unpublished observations). Therefore, the individual outcomes of an \textit{H. pylori} infection do not seem to be dependent on the LPS type expressed by the infecting strain. These results emphasize the importance of host factors in the development of \textit{H. pylori}-induced gastric ulcers.

In sharp contrast to the low levels of cytokines induced by stimulation with \textit{H. pylori} LPS, the amounts of CXC chemokines produced after stimulation were significantly increased. Chemokines are subdivided into CC chemokine

![Cytokine production by human blood monocytes after LPS stimulation.](image)

\textbf{FIG. 4.} Cytokine production by human blood monocytes after LPS stimulation. TNF-\(\alpha\), GM-CSF, and IL-10 production from human monocytes stimulated with LPS (25 \(\mu\)g/ml) from \textit{H. pylori} Hel 73, Hel 230, or Hel 234 or from \textit{E. coli}. The line in each scatter plot represents the median value. Statistical evaluations of differences between control and stimulated samples were performed by one-way ANOVA (Kruskall-Wallis) followed by Dunn’s multiple-comparison test. \(*** P < 0.001; ** P < 0.01.\)
and CXC chemokine families on the basis of the positions of their two N-terminal cysteine residues. The CC chemokines (e.g., RANTES) are active on multiple leukocyte subtypes, but they are generally inactive on granulocytes. In this study, we chose to analyze production of the CXC chemokines IL-8 and GRO-α, which preferentially recruit neutrophils, as well as the T-cell-chemotactic chemokine RANTES, which attracts both Th1 and Th2 subsets of T cells. When we analyzed supernatants from monocytes stimulated with different H. pylori LPS, we found a significant increase in CXC chemokine production but not in RANTES production. More significant, however, is our observation that the concentrations of IL-8 and GRO-α induced by H. pylori LPS were comparable to those induced by the generally more potent E. coli LPS. Our cell depletion experiments indicate that monocytes/macrophages are among the major sources of IL-8 after H. pylori LPS stimulation. Therefore, our results suggest that H. pylori LPS is an important factor in the induction of CXC chemokines and in H. pylori gastritis, and they could partly explain the continuous infiltration of granulocytes to the site of infection. The neutrophils probably also participate in further recruitment of new inflammatory cells by their own production of IL-8 (4, 12) and secretion of digested bacterial metabolites that are chemotactic for other granulocytes. In addition, TNF-α, which is produced during H. pylori infection, is a potent activator of endothelial cells and might therefore participate in the recruitment of new inflammatory cells that can participate in the eradication of the bacteria (9). The ability of H. pylori LPS to induce production of granulocyte-recruiting chemokines could also be one mechanism behind the large recruitment of inflammatory cells, especially neutrophils, to the infected meta-

plastic areas in the duodenum (18). These cells could in turn be responsible for some of the tissue damage and ulcer formation that are suggested to originate at these sites.

Previous studies have shown that H. pylori infection induces production of both neutrophil- and T-cell-recruiting chemokines in gastric mucosa as well as in vitro systems (15, 22, 35, 36, 45). In particular, a high level of IL-8 in the mucosa is a characteristic finding in H. pylori gastritis (14, 15, 36). The role of LPS in chemokine production in vivo, however, is hard to assess. In a recent study, Lindholm et al. used human stomach explants stimulated with viable H. pylori bacteria and corresponding LPS to study acute chemokine production (23a). As in our study, stimulation led to significantly increased levels of GRO-α and IL-8 that were almost as high as those induced by whole bacteria. These findings also emphasize the potential importance of H. pylori LPS for neutrophil recruitment in H. pylori-associated gastritis.

In conclusion, we have shown that H. pylori LPS is much less potent in inducing the production of proinflammatory cytokines TNF-α, IL-10, and GM-CSF by human monocytes and macrophages than are LPS from several other species. In contrast, the CXC chemokines IL-8 and GRO-α were produced in large amounts in response to H. pylori LPS, and this might be one of the factors promoting the continuous influx of neutrophils into the H. pylori-infected gastric mucosa.

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REFERENCES


3. Aspinall, G. O., and M. A. Monteiro. 1996. Lipopolysaccharides of Helico-


man peripheral mononuclear blood cells in comparison to lipopolysaccha-


11. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from hu-


13. Cover, T. L. 1997. Helicobacter pylori transmission, host factors, and bacte-


23. Lee, A. O. R., J. Ungria, Maria Corazon de Robertson, B. Daskalopoulos,


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